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Amphotericin B Formulations Exert Additive Antifungal Activity in Combination with Pulmonary Alveolar Macrophages and Polymorphonuclear Leukocytes against *Aspergillus fumigatus*

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Deoxycholate amphotericin B (DAMB) and amphotericin B lipid complex (ABLC) additively augmented the fungicidal activity of pulmonary alveolar macrophages against the conidia of *Aspergillus fumigatus*. DAMB, ABLC, and liposomal amphotericin B similarly displayed additive effects with polymorphonuclear leukocytes in damaging the hyphal elements of *A. fumigatus*.

During the past two decades, invasive pulmonary aspergillosis (IPA) has emerged as an important life-threatening opportunistic fungal infection in immunocompromised hosts (6, 9, 17, 22, 24, 26). The conidia of *Aspergillus fumigatus* enter the respiratory tract, swell, germinate, and invade pulmonary tissue as hyphae. The predominant host defenses against *A. fumigatus* in the lungs are pulmonary alveolar macrophages (PAMs) and peripheral blood polymorphonuclear leukocytes (PMNs). PAMs ingest inhaled *Aspergillus* conidia and inhibit their intracellular germination (7, 16, 23). PMNs defend the host against *A. fumigatus* by mediating damage to invading hyphae through the release of microbicidal metabolites (2, 7, 16).

Conventional deoxycholate amphotericin B (DAMB) and newer lipid formulations of amphotericin B are standard antifungal agents used in the management of IPA. Whether these compounds have a potentially beneficial additive antifungal effect in combination with host phagocytic defenses is not well understood. We therefore investigated the potential additive effects between PAMs or PMNs and conventional or lipid formulations of amphotericin B against *A. fumigatus* conidia or hyphae.

PAMs were obtained by bronchoalveolar lavage from 22 pathogen-free female New Zealand White rabbits (Hazleton, Rockville, Md.), as described previously (3). PAMs were incubated at a concentration of 10^6/ml in RPMI 1640 containing 10% fetal bovine serum (Gibco), 100 U of penicillin per ml, and 100 μg of streptomycin per ml (complete medium) at 37°C in 5% CO_2 for 2 days before the conidiocidal assay was performed (see below).

Whole blood was obtained from healthy young adult volunteers. PMNs were isolated by dextran sedimentation and Ficoll centrifugation as reported previously (15).

Strain 4215 (MYA-1163; American Type Culture Collection, Manassas, Va.), a well-characterized isolate of *A. fumigatus*, was stored, cultured, and processed for generation of conidial suspensions (12).

The amphotericin B formulations, DAMB (Bristol-Myers Squibb, Paris, France), amphotericin B lipid complex (ABLC; The Liposome Company, Princeton, N.J.), and liposomal amphotericin B (LAMB; Gilead Nextar, San Dimas, Calif.), were used at concentrations of 0.062, 0.125, and 2.5 μg/ml, respectively, in combination with PAMs and at concentrations of 0.062, 0.125, and 0.625 μg/ml, respectively, in combination with PMNs. The higher concentration of LAMB (2.5 μg/ml) was necessary in order to assess conidiocidal activity. These concentrations were selected as the most appropriate, as determined from separate dose-response conidiocidal assays and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2 *H*-tetrazolium-5-carboxanilide (XTT) experiments. In these experiments, different concentrations of drugs were mixed with the fungal targets (conidia or hyphae) and were incubated by the methods used for the assay of conidiocidal activity and the XTT assay described below. The concentrations of drugs chosen to be used in combination with phagocytes were those that achieved ≤50% activity against the fungal targets (conidia or hyphae) when they were used alone. DAMB and LAMB were provided in the form of powders and were dissolved in distilled H_2O, while ABLC was provided in aqueous solution. The antifungal drugs were added to the phagocytes simultaneously with the conidia or the hyphae.

A modified CFU assay was used to assess the conidiocidal activities of the PAMs (12). PAMs were mixed with 10^6 conidia at a 1:1 effector cell-to-target cell ratio with and without antifungal agents in a final volume of 1 ml in complete medium in polypropylene tubes. Tubes were rotated at 37°C for 6 h, PAMs were lysed with sterile water, and serial dilutions were plated as previously described (12). Colonies were counted and conidiocidal activity was calculated by the following formula: percent killing = $(1 - X/C) \times 100$, where X is the number of CFU with PAMs, antifungals, or combinations thereof at 6 h, and C is the number of CFU of conidia only at 6 h.

PMN-induced hyphal damage was assessed by use of the XTT (Sigma, St. Louis, Mo.) colorimetric metabolic assay

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TABLE 1. Conidiocidal activities produced by amphotericin B or its lipid formulations alone and in combination with rabbit PAMs against *A. fumigatus*[a]

Compound	% Conidiocidal activity		
	PAMs alone	Compound alone	PAMs + compound
DAMB	17.9 ± 5.5	14.1 ± 13.9	48.9 ± 9.6[b,e]
ABLC	21.7 ± 6.6	22.2 ± 9.7	58.3 ± 5.5[c,e]
LAMB	29.4 ± 5.8	20.6 ± 13.1	41.3 ± 6.9[d,e]

[a] The results are shown as means ± SEMs of percent conidiocidal activity for three to eight experiments for each antifungal compound.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P > 0.05$.
[e] Comparisons of antifungal compounds or PAMs and their combination were analyzed.

with coenzyme Q (2,3-dimethoxy-5-methyl-1,4-benzoquinone; Sigma) (8).

Results are shown as the means ± standard error of means (SEMs). The antifungal activities of the combinations of drugs and phagocytes were compared to the activities of each of the components alone (drug- or phagocyte-induced antifungal activities), and the resulting differences were evaluated by repeated-measures analysis of variance (ANOVA) followed by Dunnett's correction for multiple comparisons. In the case of unpaired experiments, one-way ANOVA followed by Dunnett's test was used. A two-sided P value of <0.05 indicated statistical significance.

DAMB and ABLC, but not LAMB, produced additive conidiocidal effects in combination with PAMs (Table 1). PAMs alone killed 17.9% ± 5.5% of the conidia, and DAMB alone killed 14.1% ± 13.9% of the conidia. The combination of DAMB with PAMs increased the proportion of conidia killed to 48.9% ± 9.6% ($P < 0.05$). Similarly, ABLC exhibited an additive effect on the conidiocidal activity, from 21.7% ± 6.6% and 22.2% ± 9.7% for PAMs and ABLC alone, respectively, to 58.3% ± 5.5% for their combination ($P < 0.01$). By comparison, LAMB did not exert any suppressive or additive effects on PAMs.

All of the amphotericin B formulations resulted in additive antifungal effects against *A. fumigatus* hyphae when they were used in combination with PMNs (Table 2). PMNs alone damaged 28.2% ± 8.0% of the hyphae, and DAMB alone damaged 26.7 ± 8.0% of the hyphae; the combination damaged 45.7% ± 7.0% of the hyphae ($P < 0.01$). ABLC alone damaged 49.1% ± 5.7% of the hyphae, and its combination with PMNs resulted in 60.7% ± 5.4% hyphal damage ($P < 0.05$). Likewise, the levels of hyphal damage produced by LAMB alone and by its combination with PMNs were 44.4% ± 7.3% and 61.3% ± 4.5%, respectively ($P < 0.05$).

This study found that DAMB and ABLC enhanced the fungicidal activities of PAMs against *A. fumigatus* conidia. Similarly, all three formulations of amphotericin B examined displayed additive effects with PMNs against the hyphae of *A. fumigatus*.

Previous studies have shown that voriconazole may have additive or synergistic effects with host phagocytes against *A. fumigatus* (21). However, little has been known about the interactions of amphotericin B and its lipid formulations with phagocytic host defense cells against this pathogen.

Pulmonary host defenses against *A. fumigatus* consist of PAMs, which ingest and destroy conidia, and PMNs, which damage hyphae through extracellular microbicidal mechanisms (13). Cytokines such as macrophage colony-stimulating factor, tumor necrosis factor alpha, and gamma interferon may further augment pulmonary phagocytic host defenses (3, 12, 13, 14, 27; E. Roilides, C. A. Lyman, T. Sein, and T. Walsh, Abstr. 39th Intersci. Conf. Antimicrob. Agents Chemother, abstr. 700, p. 555, 1999). Antifungal drugs that are administered to patients with IPA must act in collaboration with host phagocytes in the microenvironment of infected tissues. This collaboration must occur either extracellularly in the case of hyphae or intracellularly (within the phagocytes) in the case of conidia.

The proposed mechanisms of action of amphotericin B include the formation of transmembrane pores, induction of lipoperoxidation, inhibition of membrane enzymes, blockade of endocytosis, and stimulation of phagocytic cells (1, 5, 28). Amphotericin B in the form of DAMB may increase the killing of phagocytosed *A. fumigatus* conidia by macrophages (4), inhibit PMN migration and chemotaxis (15, 19, 29), enhance PMN adherence, and reduce PMN viability (15, 18, 20, 29). At the concentrations at which they were used in the present study, none of the antifungal agents exerted deleterious effects on the antifungal activities of PAMs or PMNs against conidia or hyphae of *A. fumigatus*, respectively.

There are several potential mechanisms for the enhanced antifungal activities of amphotericin B formulations used in combination with PAMs or PMNs observed in this study. First, amphotericin B may induce an increase in fungal membrane permeability to PMN microbicidal products such as oxidative burst metabolites or nonoxidative products. Second, amphotericin B may induce the secretion of oxidative and nonoxidative metabolites by PMNs. In this regard, amphotericin B is known to induce secretion of immunoenhancing cytokines such as interleukin-1 and tumor necrosis factor alpha by

TABLE 2. Hyphal damage produced by amphotericin B or its lipid formulations alone and in combination with human PMNs against *A. fumigatus*[a]

Compound	% Hyphal damage		
	PMNs alone	Compound alone	PMNs + compound
DAMB	28.2 ± 8.0[c]	26.7 ± 8.0[c]	45.7 ± 7.0[c,d]
ABLC	28.2 ± 8.0[c]	49.1 ± 5.7[b]	60.7 ± 5.4[b,c,d]
LAMB	28.2 ± 8.0[c]	44.4 ± 7.3[b]	61.3 ± 4.5[b,c,d]

[a] A 200-μl suspension of 1.5×10^4 conidia in yeast nitrogen broth> was incubated in flat-bottom cell culture microplates at 32°C for 18 to 20 h. The yeast nitrogen broth> was then replaced by Hanks balanced salt solution, antifungal drugs, and PMNs at a 5:1 effector cell-to-target cell ratio. After incubation at 37°C in 5% CO_2 for 2 h and cell lysis> by washing with H_2O three times and shaking at room temperature for 5 min, 150 μl of phosphate-buffered saline containing 0.25 mg of XTT per ml and 40 μg coenzyme Q per ml was added. After incubation for an additional 60 min, 100 μl was transferred and read in a spectrophotometer (450 nm). Percent hyphal damage was calculated as $(1 - X/C) \times 100$, where X and C are the optical densities of test wells and control wells with hyphae only, respectively. The results are shown as means ± SEMs of percent hyphal damage for seven duplicate experiments for each antifungal compound.
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] Comparisons of antifungal compounds or PMNs and their combination were analyzed.

phagocytic cells (11). Increased expression of tumor necrosis factor alpha further enhances the host response to *A. fumigatus* (12). Third, the enhanced conidiocidal activities of PAMs against *A. fumigatus* conidia induced by DAMB and ABLC may be due to enhanced phagocytosis of conidia or enhanced oxidative and nonoxidative mechanisms (25, 27).

LAMB appears to interact with host phagocytes differently from the other amphotericin B formulations by acting synergistically only with PMNs against *A. fumigatus* hyphae. A different configuration of amphotericin B in the liposomes of LAMB may be responsible for the lack of additive effects of the latter when it is used in combination with PAMs. In the case of ABLC, amphotericin B is concealed within ribbonlike structures. The release of lipases by the fungus breaks down the ribbonlike structures, thus releasing drug (10) and increasing its local concentration. This could enhance the local antifungal activity of amphotericin B and increase the permeability of the fungal membrane to PMN fungicidal products. LAMB is a small (80-nm) negatively charged liposome, while ABLC is a lipid complex that may be as large as 1,600 nm. A small negatively charged liposome like LAMB is less avidly ingested by macrophages, in contrast to the large lipid complex, which is more rapidly taken up by phagocytes. The higher initial intracellular concentrations of ABLC would be more likely to exert a conidiocidal effect in synergy with PAMs than LAMB would because LAMB would likely remain predominantly extracellular. The relative lack of synergy between LAMB and PAMs may also have been related to the relatively high levels of activity of the PAMs observed in these experiments. Indeed, a trend consistent with that observed with DAMB and ABLC also was noted for LAMB, suggesting that some interaction also may occur between LAMB and PAMs.

In conclusion, this study found that DAMB and ABLC act synergistically with both types of pulmonary phagocytes (PAMs and PMNs) and that LAMB acts synergistically principally with PMNs, demonstrating that certain antifungal agents may act synergistically with phagocytes during the early (conidial) and/or late (hyphal) phase of pulmonary host defense in response to *Aspergillus.*

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